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Raman spectroscopy analysis of dental enamel treated with whitening product – Influence of saliva in the remineralization

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ABSTRACT

In this work we present the analysis of dental enamel treated with an over-the-counter whitening product, bought in e-commerce at a very low cost, used without medical supervision in an abusive manner, in order to evaluate its demineralization action. Moreover, we studied the influence of renewal or non-renewal of saliva solution in which the specimens were stored throughout the study.

The Degree of Demineralization was determined through the evaluation of the PO_4^{3-} symmetric stretching band (~959 cm^{-1}) in Raman spectra of the specimens in different days during the course of the study. Results showed that a maximum of demineralization occurred between days 27 and 34 of application.

Titration of the whitening product revealed a content of hydrogen peroxide 170-fold higher than what is allowed in Europe, according with legislation. Despite this extreme concentration of hydrogen peroxide, the demineralization was not as great as could be expected suggesting an important role of the pH of the solution in this demineralization mechanism.

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1. Introduction

In the last decades, patients' demand for aesthetic dental procedures including bleaching has risen considerably [1]. This fact fueled the development of the tooth whitening industry with the appearance of different types of products and techniques. Moreover, patients' expectations for an easy to apply, cheap, effective, fast and minimally invasive bleaching method are at its highest peak, and drove the market emergence of a new class of over-the-counter sold products. These products are low cost marketed, sold without any medical prescription or restriction even online, despite the fact of frequently presenting active agent concentrations well above the European Commission safety levels [1]. The use of these products without medical supervision, may result in exposure of individuals to bleaching agent intake and cause severe lesions in the oral cavity, such as tooth sensitivity, reduction of microhardness and increase of dental enamel roughness [2].

The majority of these bleaching products include hydrogen peroxide (HP) or its stabilized form, carbamide peroxide (CP) as an active principle. HP has low molecular weight, allowing it to diffuse in the tooth structure and trigger a series of oxidative reactions. This agent decomposes in water and produces unstable and reactive free radicals, such as oxygen which then react with the unsaturated organic molecules responsible for dental tissue color changes, resulting in smaller, lighter and reflective molecules. Consequently, these structures cause the tooth to appear brighter. However, whether these bleaching reactions may alter the enamel composition has been object of study in the past. It is accepted that under supervised clinical conditions, dental bleaching is safe for enamel integrity but less is known about abusive and/or over-the-counter use.

Raman spectroscopy has been widely used in determining the changes at the molecular level of inorganic matter of mineralized tissues of teeth with many advantages over other methods of analysis. As a non-destructive technique, it allows the analysis of samples before and after applying the tooth whitening product making it a very suitable technique in self-controlled studies [3].

Silveira et al. [4] used confocal micro Raman technique to identify the presence of oxygen generated by tooth bleaching products in tooth enamel surface, supporting the theory that oxygen remains in tooth mineralized tissues following some dental treatments and thus reducing adhesive forces and compromising the restorative treatments.

Raman spectroscopy has been used to study the changes in the symmetric stretching mode of the tetrahedral phosphate (PO_4^{3-}) group which is representative of the mineral phase (carbonated hydroxyapatite) in teeth. This has been done with teeth treated with different concentrations of bleaching agents as well as different storing methodologies for the specimens [4–8].

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An investigation developed by Santini et al. [5] used 10% CP as bleaching agent applied in tooth enamel during 28 days, while the specimens were stored in buffered saline, which was changed daily.

Vargas-Koudriavtsev et al. [7] used more concentrated, dentist supervised night guard bleaching gels, with varying HP concentrations (9.5% HP, 38% CP and 14% HP) according to the manufacturer's instructions (also during 28 days) and stored in distilled water.

Castro et al. [6] used two over-the-counter bleaching gels (11% HP, pH 4 and 14% HP, pH 3), applied exceeding the manufacturer's recommendations, storing the specimens in human saliva throughout the study, without renewal.

In all these *in vitro* studies, a positive correlation was obtained between the concentration of the bleaching agent and the decrease of phosphate concentration, hence a demineralization effect on the enamel. There is, however, a proven re-mineralization effect of the saliva that needs to be taken in consideration when generating translational knowledge to clinical cases [4].

However, the contribution of the pH of the bleaching gel needs further assessment, as it is normally not determined, or determined but its effects are not discussed [8].

The aim of this *in vitro* study was to evaluate the effects of an over-the-counter tooth whitening product (Easy White 44%) and storage medium conditions in teeth mineralization. The rationale was to perceive if the changing of the storage media influences the effects of the bleaching gel in the dental tissues, because this will lead to the construction of better *in vitro* study models.

2. Materials and Methods

2.1. Specimen Preparation

Six anterior healthy teeth, extracted for periodontal or orthodontic reasons, from the LIBPhys-FMDUL tooth bank, were selected and preserved in a 0.5% (w/w) chloramine solution for no longer than 6 months. The samples were selected by experts and the exclusion criteria would be the presence of lesions, cavitated or not, including white spots. Intrinsic demineralization that could not be assessed in the clinical practice by experts could not be accounted for. Hydroxyapatite is an anisotropic material, thus, signal intensity might be affected by the crystallographic orientation. According to the research of Pezzotti et al. [9,10] this deviation in the orientation is enhanced for molar teeth and mainly in the occlusal region. This way, and in order to minimize the effects of crystallographic orientation the samples were obtained only from the vestibular surface of incisor teeth with a precision diamond saw (Buehler Isomet 1000, USA) in order to obtain 12 samples with 8 mm × 2 mm and only with dental enamel. Afterwards, the samples were stored in storage vials in a new chloramine solution until the beginning of the application of the whitening products and from there properly identified.

Specimens were randomly divided into two groups with GraphPad Quickcals software. In group A, artificial saliva was renewed daily (A1 to A6); in group B artificial saliva was kept unchanged along the entire study (B1 to B6).

2.2. Artificial Saliva

Artificial saliva (Artificial Saliva Gal Fovet-SAGF) used as storage medium was a solution without protein and organic compounds (except for urea and/or organic acids for pH adjustment). The compound and its concentration is listed in Table 1. The measured pH for the SAGF saliva was 7.1 [11].

2.3. Whitening Products

Easy White 44% Carbamide Peroxide (Easy-Whitening LLC, USA) over-the-counter was acquired in a e-commerce website, in European Union. According to the manufacturer the active agent concentration is 44% of CP and the gel should be applied for 30 min, not exceeding 14 days.

Table 1
Composition of artificial saliva used in this study.

	Concentration (mg/L)
NaCl	125.6
KCl	963.9
$CaCl_2 \cdot 2H_2O$	227.8
KH_2PO_4	654.5
Urea	200
NH_4Cl	178
$NaHCO_3$	630.8
KSCN	189.2
$Na_2SO_4 \cdot 10H_2O$	763.2

In order to verify if the concentration of active agent corresponded to that indicated by manufacturers, a cerium sulphate titration was performed, which allowed the percentage of hydrogen peroxide in the product to be calculated [12,13].

Furthermore, the bleaching gel was examined using Raman spectroscopy and its pH was measured by using a pH meter, the BASIC 20 (Crison Instruments, Spain) with 0.01 resolution. Three measurements were performed and the mean value was determined.

2.4. μ-Raman Confocal Spectrometer

Raman spectra of samples were obtained using a Horiba XploRA Confocal Microscope using the near infrared laser (785 nm). Because the main goal was to evaluate the intensity of the band at ~959 cm^{-1}, spectra were acquired with only one window centered at 900 cm^{-1}, using a 1200 lines/mm grating. This way, the spectral range investigated was from 400 cm^{-1} to 1500 cm^{-1} with spectral resolution of 4 cm^{-1}. Only for the whitening product, the spectral range was from 400 cm^{-1} to 1800 cm^{-1}.

A 100× (N.A. = 0.9) objective was used as well as a 50% neutral density filter rendering a spatial resolution of 1 μm and an incident power on the sample of 5.0 ± 0.4 mW (lasercheck®, Edmund optics). Using an entrance slit of 100 μm, and a confocal hole of 300 μm, the scattered light collected by the objective was dispersed onto the air cooled CCD array of an Andor iDus detector. Energy calibration and band intensity were measured in the beginning of each measurement campaign using a Si-wafer standard.

The exposure time was 5 s and the accumulation number was 10 times for each window. For each sample, an average of 10 measurements was performed. Spectra deconvolution has been performed using the software LabSpec (v5.58.25, Horiba, France), making use of a polynomial baseline correction to remove the background due to fluorescence. The intensities were determined by integrating the area under the bands.

The intensity of PO_4^{3-} band was determined in order to calculate the Degree of Demineralization (DD), according to the following formula [14]:

$$DD\ (\%) = \left(1 - \frac{I\ demineralized}{I\ mineralized}\right) \times 100$$

where $I_{mineralized}$ is the intensity of the hydroxyapatite peak at ~959 cm^{-1} in the mineralized enamel (control), and $I_{demineralized}$ is that measured for each day of application.

2.5. Methodology

Because the aim of the study was to investigate the effects of unsupervised over-the-counter whitening products in dental enamel, an abusive protocol exceeding the manufacturer's recommendations was

developed. The treatment consisted of covering the specimens with the bleaching gel for 30 min daily for a period of 44 days. Between the applications, samples were washed with distilled water, brushed with a toothpaste fluoride free and stored in appropriated vials. In group A, artificial saliva was renewed daily (A1 to A6); in group B artificial saliva was kept unchanged along the entire study (B1 to B6).

The study was undertaken in a self-control approach, so measurements were performed before the first application of the beaching gel (control) and at 7, 10, 14, 17, 20, 27, 30, 34, 37, 41, 44 days of application. To further evaluate an eventual remineralization effect of saliva, measurements were also performed one and two weeks after the end of the treatment.

2.6. Statistical Analysis

Data and analyses were computed using a computer statistical package (SPSS v.23, SPSS Inc., Chicago, IL, USA). Discrete data were analyzed using Fisher exact test and direct 95% confidence interval analysis. The precision of the measurements was expressed as the Pearson coefficient variation in percentage. Mean values of degree of mineralization of enamel were analyzed with one-way ANOVA with repeated measures.

The objectives of this study were:

- to evaluate the initial content of active principle of the bleaching product and compare it with the manufacturer claimed content.
- to evaluate the Degree of Demineralization of teeth, treated with an over-the-counter product, exceeding the manufacturer's recommendations and;
- to evaluate the influence of the storage medium conditions in teeth demineralization degree.

The study hypothesis is:

- There is a significant difference between the degree of demineralization of enamel (relative %) following tooth bleaching procedure and saliva storage in artificial saliva for two weeks or daily change of medium storage.

3. Results

Titrations were performed in order to confirm the HP concentration of the whitening product. It was determined that the product has a HP concentration of 17.01% corresponding to a CP concentration of 46.99% [15]. This concentration is statistically significantly higher than that indicated by the manufacturer for this product ($p < 0.05$). Regarding the pH of the product, measurements with the pH meter revealed a value of 5.11 ± 0.02.

Fig. 1 displays the Raman spectrum obtained for a drop of whitening product where the four bands at 1003, 1461, 1600 and 1664 cm^{-1}, identify the urea molecule [16] in solution. This molecule and the HP (at 878 and 1392 cm^{-1}) resulting from degradation of CP, confirm that the latter was the bleaching agent present in the product. Moreover, characteristic bands of the products excipients, propylene glycol (525, 803 and 841 cm^{-1}) [17] and glycerol (417, 483, 678, 928 and 1045 cm^{-1}) [18]. These excipients have mainly a thickening function. Carbopol, used frequently for on-off release function of the product, was not indubitably identified, although bands at 525 and 878 cm^{-1} might be indicative of its presence [19].

Fig. 2a presents a Raman spectrum obtained during this work. The characteristic bands of hydroxyapatite are assigned in Table 2 [20,21]. The band situated at ~959 cm^{-1} corresponding to the symmetric P—O stretching vibration of phosphate ions, experienced variation throughout the study as can be seen, in Fig. 2b depicting the comparison of the spectra obtained for sample B2 before treatment (control) and at day14, day27, day30, day37 and day44 after background correction.

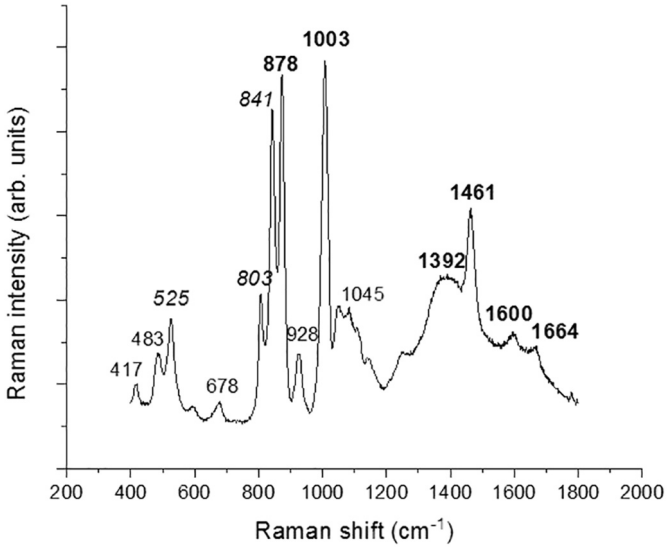

Fig. 1. Raman spectrum obtained for a drop of whitening agent.

Changes in band position are well within the resolution of the detector, so no further inferences can be made on the subject. Regarding the calculated Degree of Demineralization (DD), Fig. 3 shows the column chart with the comparison of the mean DD throughout the study for group A and B. No significant differences were found between both groups.

In group A, at the end of the period recommended by the manufacturer for the duration of treatment (14 days), the average value of the Degree of Demineralization of the samples was 5.0 ± 1.5%. Extending the application of the product beyond recommended, there is a maximum demineralization for all samples between day27 and day34. Table 3 represents the value for the maximum DD for each sample of this group in the study.

After this maximum of demineralization, all samples undergo a gradual recovery of their degree of mineralization, followed by a new trend of increased demineralization to occur on day41 or day44.

In group B the average value of the Degree of Demineralization after 14 days of treatment was 4.6 ± 3.2%, and the maximum demineralization occurred between day27 and day34 for all samples. Table 3 represents the maximum value of DD for each sample of this group in the

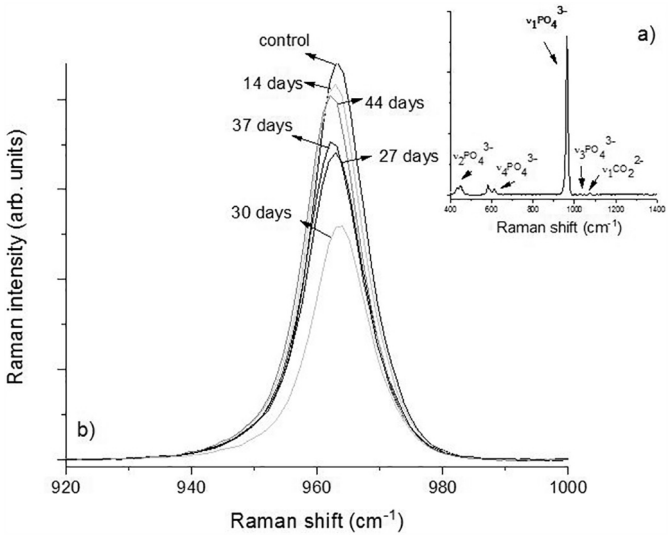

Fig. 2. a) Raman spectrum obtained for control sample B2; b) comparison of the baseline-corrected spectra obtained for sample B2, before treatment and at days 14, 20, 27, 30, 37 and 44.

Table 2
Assignment of the characteristic bands of hydroxyapatite for the spectrum in Fig. 2 [19,20].

Band (cm^{-1})	Assignment
431, 449	Doubly degenerate bending mode (ν_2) of the PO_4^{3-} group (O—P—O) bond
582, 595, 612	Triply degenerate bending mode (ν_4) of the PO_4^{3-} group (O—P—O) bond
959	Symmetric stretching mode (ν_1) of the PO_4^{3-} group P—O bond
1027, 1047, 1075	triply degenerate asymmetric stretching mode (ν_3) of the PO_4^{3-} group P—O bond
1072	symmetric stretching mode (ν_1) of the CO_3^{2-} group

study. Similarly, in this group, after reaching its maximum demineralization, all samples have undergone a gradual recovery in their degree of mineralization.

In Fig. 4, the spectra obtained for control, day7, day27, day44 are presented without background correction. A decrease in fluorescence is clearly observed throughout the study, suggestive of a loss of organic content by the samples.

4. Discussion

This study aimed to evaluate teeth's DD in different storage conditions, following a simulated overuse of an over-the-counter dental bleaching product. The data obtained shows that the current protocol, does not result in an increased DD, independently of the medium storage conditions. The relatively high Standard Deviation obtained for each day is related with biological diversity between samples of the same group. However, and despite the fact that no significant differences were found between group A and B, the Pearson coefficient of variations in the saliva renewal group are smaller and without variation along the time when compared with those in the non renewal group which increased overtime (Table 3). This suggests that a greater dispersion and variability exists when saliva is not changed, leading to lower precision values of the measurements suggestive that saliva renewal has a greater cost/benefit ratio.

Dental bleaching product usually presents HP or CP as active principle. The HP is very unstable in the presence of water and basic pH. Commonly, dental material manufacturers present the commercially available product with an acidic pH, in order to preserve it and increase the product's shelf life. In fact, the pH registered for the product tested was 5.11. Considering the pH for demineralization of hydroxyapatite (approximately 5.5), which is the dental enamel inorganic matter

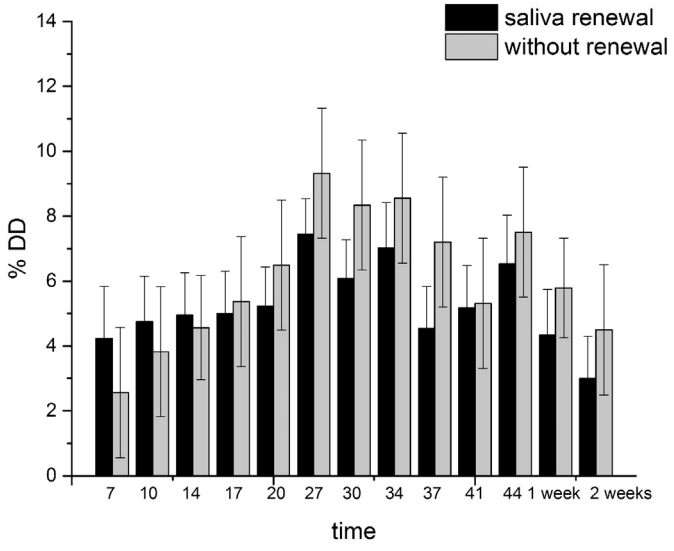

Fig. 3. Mean Degree of Demineralization (DD) and standard deviation obtained for group A and B samples.

Table 3
Mean degree of demineralization, standard deviation (SD) and coefficient of variation (CV) for group A and B. Notice, a substantially higher CV when storage medium is not exchanged daily (group B).

	Group A - saliva change			Group B- no saliva change		
	Mean	SD	CV	Mean	SD	CV
d7	4.2	2.3	0.53	2.6	2.6	1.01
d10	4.2	2.1	0.50	3.8	2.8	0.74
d14	5.0	1.5	0.31	4.6	3.2	0.70
d17	5.0	1.8	0.36	5.4	6.1	1.14
d20	5.2	1.7	0.32	6.5	5.4	0.83
d27	7.4	1.9	0.26	9.3	5.5	0.59
d30	6.1	2.1	0.35	8.3	5.9	0.71
d34	6.1	3.5	0.58	8.6	7.0	0.81
d37	4.5	1.9	0.42	7.2	5.6	0.78
d41	5.2	2.4	0.47	5.3	3.8	0.71
d44	6.5	2.1	0.33	7.5	2.6	0.35

structural unit, concerns regarding the effect of this products in oral tissues, are reasonable and required extensive study [22].

Furthermore, dental enamel is an epithelial origin tissues that lacks the presence of cells to ensure an effective turnover [23]. The loss of tissue due to demineralization would result in a permanent loss. Fortunately, the presence of saliva in oral cavity, with its high buffer capacity and supersaturated composition in phosphate and calcium, minimizes these permanent deleterious effects [24]. However, this buffer and remineralization effect is limited. In order to test this limitation, and to optimize further research, we performed the current study with two groups: one with a daily medium storage saliva exchange, and a second group, without exchange of the medium during the whole study. The results obtained show that, for the duration, sample size and conditions of this study, the daily exchange of artificial saliva doesn't affect significantly the DD of samples. After the dental bleaching protocol, during 44 days, the remaining saliva continues to present some remineralization capacity elucidated by a decrease in DD. The difference between groups was not statistically significant. However, considering the Pearson coefficient variation (Table 2), a higher variation in group B was obtained. The results suggest that the daily exchange of storage medium (saliva) should be performed in order to narrow the measurements variation. This effect should be studied in a more extensive way, namely by a higher sample size.

We used an artificial saliva (SAGF) without protein or glycoprotein prepared with a well-established composition as expressed in Table 1. Human saliva could be used and easily obtained, but using it would create a wider range of composition. Even though it would better simulate *in vivo* conditions, we would lose control of variables. With artificial

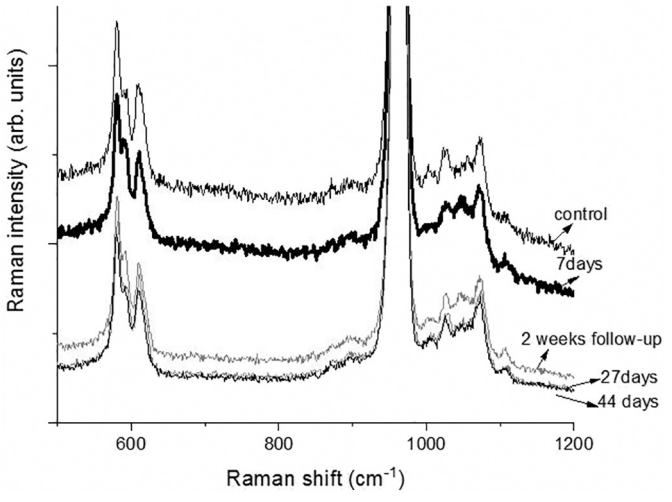

Fig. 4. Comparison of the Raman spectra background obtained for sample A3, without baseline correction, before treatment and at days 7, 27, 44 and 2 weeks follow-up.

saliva, the conditions are better established and defined, reducing the variability that could interfere with the measurements. Besides this, presence of abundant organic material in enamel surface and eventual dental plaque with enormous bacterial amounts could interfere severely with the fluorescence registered.

The manufacturer of this tooth bleaching agent claims that this product contains 44% CP as active agent for tooth bleaching. In order to test its veracity, a colorimetric titration using cerium sulphate was performed [25]. This procedure resulted in a total amount of 17.01% of HP, the equivalent to 46.99% CP according to the conversion rate [15]. The presence of a higher than claimed HP concentration appears to be a common practice among dental product manufactures [26]. The reason for this repeated findings remain unclear. These findings, related not only to a significantly higher concentration of active principle but also to a substantially higher content than allowed by European Directive 2011/84/EU, that restrain the commercially available products over-the-counter for dental bleaching, to a maximum concentration of 0.1% HP [27]. These findings raise concerns regarding oral health of patients, since these products are easily available to consumers, who tend to perform this medical treatment without any medical supervision. This lack of supervision may result in an increase of free radicals contacting the healthy oral soft tissues with all the concerns related to oxidation of organic tissues such as the Fenton reaction [28]. To prevent this deleterious effect, an increased control of over-the-counter products for tooth bleaching, namely considering the active principle concentration are needed and desirable.

Regarding the decrease in fluorescence observed throughout the study, it is suggestive of a loss of organic content by the samples. This reduction of fluorescence and consequently of organic matter is relevant, as the presence of the latter is necessary to retain water and somehow responsible for tooth color. Finally, in the analysis at 2 weeks follow-up the increase of fluorescence is again noticed, corroborating the behavior of a gradual recovery in the DD. These results are consistent with previous studies [4,6,10,29,30]; what should be further clarified is if this reduction causes permanent optic modifications. Comparing the obtained results with the study by Castro et al., [6] also using over-the-counter products in an abusive manner, it is clear that a more severe demineralization of the enamel is reached when using products with much lower pH, namely a DD up to 65% after using a bleaching product with pH = 3, followed by an increase of DD. Similarly, Pezzotti et al. [10] determined a non-monotonic behavior of the intensity of the symmetric P—O stretching vibration of phosphate, attributed to the removal of enamel material due to the exposure to acidic beverage (pH = 2.5).

5. Conclusions

In this work we have studied the Degree of Demineralization (DD) of an over-the-counter whitening gel, applied in an abusive manner. The results show a tendency to a higher demineralization of the enamel after 27 to 30 days of application, however the differences in mean DD values, for all samples, between measuring days are not statistically different considering the variability within samples. Moreover, we aimed at exploring if the renewal or not of the storage medium in similar *in vitro* studies would influence the outcome of the investigation. From the obtained results, the medium storage conditions tested do not result in statistically significant differences in the DD values. Moreover, it is important to highlight that other substances present in the OTC product apart from the CP might contribute to the overall acidity of the resulting gel, so the whole product must be considered when performing *in vitro* studies.

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